METHODS OF DETERMINING EFFICACY OF TREATMENTS OF DISEASES OF THE BOWEL

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CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation in part of US Application No. 10/404512, filed April 1, 2003.

TECHNICAL FIELD

The present invention relates to the field of inflammatory diseases of the bowel, particularly to methods for screening and determining efficacy of treatments of these diseases. Furthermore, kits to carry out the method of the present invention are also provided

BACKGROUND

Inflammatory diseases of the bowel is the general term for diseases that cause inflammation of the intestines such as irritable bowel syndrome (IBS), and the inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease that are chronic inflammatory disorders of the gastrointestinal (GI) tract. For example, ulcerative colitis is an IBD that causes inflammation of the mucosa lining of the large intestine, usually occurring in the rectum and lower part of the colon, but it may affect the entire colon. Crohn's disease may affect any section of the GI tract (i.e. mouth, oesophagus, stomach, small intestine, large intestine, rectum and anus), and may involve all layers of the intestinal wall. The cause of many of these diseases is unknown.

IBS is a functional gastrointestinal disorder in which abdominal discomfort or pain is associated with defecation or change in bowel habit, and with features of disordered defecation. Theses symptoms represent a condition in which disturbances in motor and/or sensory function of the gut may be associated with psychosocial disorders, and the interaction leads to symptoms at several levels of the gastrointestinal tract.

IBS is now considered to be the most common gastrointestinal disorder. Prevalence in western world is estimated to be 15-20% of the adolescent and adult population and the disorder accounts for 20-50% of the referrals to gastroenterology clinics.

Current approaches to management of IBS consist of identification of symptoms consistent with the syndrome and the exclusion of organic disease with similar presentation, followed by non-pharmacological and pharmacological therapies, where appropriate. Current pharmacological therapeutic options are limited and the effectiveness of many is poorly documented. The current pharmacological therapies aim at treating symptoms with the rationale being either to modulate intestinal motility, decrease visceral sensitivity or treat associated disorders, particularly anxiety or depression.

The most common symptoms of IBD include abdominal pain, tenesmus, fecal urgency and bloody diarrhoea. Sufferers may also experience fatigue, weight loss, loss of appetite, rectal bleeding and loss of body fluids and electrolytes. The symptoms of the disease are usually progressive, and sufferers typically experience periods of remission followed by severe flare-ups.

Despite the prevalence of IBD (it affects an estimated 2 million people in the United States alone), there is no cure and the exact causes of the disease are not yet understood. Conventional treatments for IBD have involved anti-inflammatory drugs, immunosuppressive drugs and surgery. However, many of the drugs used for treating the disease have negative side effects such as nausea, dizziness, anaemia, leukopaenia, skin rashes and drug dependence, and the surgical procedures are often radical procedures, such as intestinal resectomy and colectomy.

This has led to several investigators to attempt to identify new and novel drugs for treatment of the inflammatory diseases of the bowel. Unfortunately, the very nature of the disease means that screening and measuring the efficacy of potential treatments in human subjects is very difficult. Often, the results of human trials depend upon subjective testimony from the trial candidates themselves, with little or no biochemical or physiological data to substantiate claims. Animal models may be used to allow tissue sections from affected organs to be taken, but drugs effective in animal models do not always have the same efficacy in humans.

The control of inflammatory diseases is exerted at a number of levels. The controlling factors include hormones, prostaglandins, reactive oxygen and nitrogen intermediates, leukotrienes and cytokines. Cytokines are low molecular weight biologically active proteins that are involved in the generation and control of immunological and inflammatory responses. A number of cell types produce these cytokines, with neutrophils, monocytes and lymphocytes being the major sources during inflammatory reactions due to their large numbers at the injured site.

Multiple mechanisms exist by which cytokines generated at inflammatory sites influence the inflammatory response. Chemotaxis stimulates homing of inflammatory cells to the injured site, whilst certain cytokines promote infiltration of cells into tissue. Cytokines released within the injured tissue result in activation of the inflammatory infiltrate. Most cytokines are

pleiotropic and express multiple biologically overlapping activities. Cytokine cascades and networks control the inflammatory response, rather than the action of a particular cytokine on a particular cell type. As uncontrolled inflammatory responses can result in diseases such as inflammatory diseases of the bowel, it is reasonable to expect that cytokine production has gone astray in individuals affected with these diseases. However, as many cytokines may have both pro- and anti-inflammatory activities, it is very difficult to attribute disease symptoms, or recovery there from, with a particular individual cytokine.

Based on the forgoing, it is desirable to provide methods for screening and measuring the efficacy of potential treatments for inflammatory diseases of the bowel in humans or other mammals that generate biochemical or physiological data. This data could be used to evaluate the efficacy of the treatment. It is further desirable to provide methods for measuring changes in the levels of specific cytokines potentially involved in the pathogenesis of inflammatory diseases of the bowel such that the prognosis and disease progression of a subject with inflammatory diseases of the bowel can be monitored.

SUMMARY

The present invention provides novel methods for determining the efficacy of a treatment of inflammatory diseases of the bowel in mammals *in vivo* comprising the steps of:

a) measuring the level of at least one anti-inflammatory cytokine and at least one pro-inflammatory cytokine in a biological sample from a mammalian subject;

b) determining the ratio of the at least one anti-inflammatory cytokine to the at least one pro-inflammatory cytokine;

c) administering said treatment;

d) measuring the level of the at least one anti-inflammatory cytokine and the at least one pro-inflammatory cytokine in a biological sample from said mammalian subject at a time following administration of said treatment;

e) determining the ratio of the at least one anti-inflammatory cytokine to the at least one pro-inflammatory cytokine following administration of said treatment;

wherein, an increase in the ratio of anti-inflammatory cytokine to pro- inflammatory cytokine following the administration of said treatment is indicative of an inhibitor of inflammatory diseases of the bowel, and no change or a decrease in the ratio of anti-inflammatory cytokine to pro-inflammatory cytokine following the administration of said treatment is indicative of said treatment not being an inhibitor of inflammatory diseases of the bowel.

In addition, the present invention provides *in vitro* methods for screening compositions for efficacy in the treatment of inflammatory diseases of the bowel comprising the steps of:

a) providing a biological sample comprising at least one gut-derived cell type;

b) treating said biological sample with the composition *in vitro*;

c) measuring the level of at least one anti-inflammatory cytokine and at least one pro-inflammatory cytokine in the biological sample at a time following treatment with the composition;

d) determining the ratio of the at least one anti-inflammatory cytokine to the at least one pro-inflammatory cytokine in the biological sample at a time following treatment with the composition;

characterised in that a ratio as determined in step (d) from the treated biological sample greater than the same ratio determined in an untreated control biological sample tested concurrently is indicative of the composition being an inhibitor of inflammatory diseases of the bowel, and a ratio as determined in (d) the same as or less than the untreated control biological sample ratio is indicative of the composition not being an inhibitor of inflammatory diseases of the bowel.

The present invention is also directed towards providing further use of the methods herein, and kits for carrying out the methods herein.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a bar graph demonstrating the mean ratio of IL-10 to IL-12 generated from PBMCs from IBS patients with *in vitro* stimulation both pre- and post-feeding with *Bifidobacteria infantis*.

Fig. 2 is a bar graph demonstrating the mean ratio of IL-10 to TNF-□ generated from PBMCs from IBS patients with *in vitro* stimulation both pre- and post-feeding with *Bifidobacteria infantis*.

Fig. 3 is a bar graph demonstrating the mean ratio of IL-10 to IFN-□ generated from PBMCs from IBS patients with *in vitro* stimulation both pre- and post-feeding with *Bifidobacteria infantis*.

Table 1 demonstrates the Pearson's correlation coefficients and *p*-values for testing the statistical significance of the negative association between the change in IL-10 to IL-12 ratio and the change in abdominal pain/discomfort score in IBS patients fed with *Bifidobacterium infantis*. Note that the mean IL-10 to IL-12 ratio increased and the mean abdominal pain/discomfort score decreased from pre- to post-feeding with *Bifidobacterium infantis*.

Figure 4 is a bar graph that demonstrates the ratio of anti-inflammatory cytokines to pro-inflammatory cytokines produced by mesenteric lymph node cells (MLNCs) stimulated *in vitro*.

Figure 5 is a bar graph that demonstrates the ratio of anti-inflammatory cytokines to pro-inflammatory cytokines produced by Peyer's patch cells (PPs) stimulated *in vitro*.

Figure 6 is a bar graph that demonstrates the ratio of anti-inflammatory cytokines to pro-inflammatory cytokines produced by mesenteric lymph node cell-derived dendritic cells (MLNC DCs) stimulated *in vitro*.

DETAILED DESCRIPTION OF THE INVENTION

All weights, measurements and concentrations herein are measured at 25°C on the composition in its entirety, unless otherwise specified.

Unless otherwise indicated, all percentages of compositions referred to herein are weight percentages and all ratios are weight ratios.

Unless otherwise indicated, all molecular weights are weight average molecular weights.

Unless otherwise indicated, the content of all literature sources referred to within this text are incorporated herein in full by reference.

Except where specific examples of actual measured values are presented, numerical values referred to herein should be considered to be qualified by the word "about".

As used herein, "inflammatory diseases of the bowel" include "irritable bowel syndrome - IBS" and "inflammatory bowel disease – IBD".

As used herein, "inflammatory bowel disease" or "IBD" includes diseases that cause inflammation of the intestines such as ulcerative colitis and Crohn's disease.

Methods and Use

The present invention is directed towards providing methods of determining efficacy of a treatment of inflammatory diseases of the bowel *in vivo* in mammals comprising the steps of:

a) measuring the level of at least one anti-inflammatory cytokine and at least one pro-inflammatory cytokine in a biological sample from a mammalian subject;

b) determining the ratio of the at least one anti-inflammatory cytokine to the at least one pro-inflammatory cytokine;

c) administering said treatment;

d) measuring the level of the at least one anti-inflammatory cytokine and the at least one pro-inflammatory cytokine in a biological sample from said mammalian subject at a time following administration of said treatment;

e) determining the ratio of the at least one anti-inflammatory cytokine to the at least one pro-inflammatory cytokine following administration of said treatment;

wherein, an increase in the ratio of anti-inflammatory cytokine to pro- inflammatory cytokine following the administration of said treatment is indicative of an inhibitor of inflammatory diseases of the bowel, and no change or a decrease in the ratio of anti-inflammatory cytokine to pro-inflammatory cytokine following the administration of said treatment is indicative said treatment not being an inhibitor of inflammatory diseases of the bowel. The method of the

present invention comprises measuring and determining the ratio of at least one anti-inflammatory cytokine and at least one pro-inflammatory cytokine before the treatment is administered. This may include determining the levels and ratios therein at least once before treatment is commenced, ideally at day 0, immediately before the treatment is commenced. Alternatively, the levels and ratio of said cytokines may be determined repeatedly over a given time before the commencement of treatment in order to provide a baseline measurement as known to those skilled in the art. The method of the present invention may further comprise repeating steps (d) and (e) at least once at similar time points following the administration of said treatment. Furthermore, the method of the present invention may comprise repeating steps (d) and (e) at least once at similar time points whilst said mammalian subject is still being administered said treatment. The method of the present invention may be utilised to both screen and clinically evaluate unknown or new treatments or compositions for efficacy in the treatment of inflammatory diseases of the bowel. Furthermore, the method of the present invention may also be used to monitor the efficacy of a known treatment in an individual patient with inflammatory disease of the bowel. Further still, the method herein may be used to provide a predictive biomarker for inflammatory diseases of the bowel helpful in diagnosis of the disorder.

In addition, the present invention provides *in vitro* methods for screening compositions for efficacy in the treatment of inflammatory diseases of the bowel comprising the steps of:

a) providing a biological sample comprising at least one gut-derived cell type;

b) treating said biological sample with the composition *in vitro*;

c) measuring the level of at least one anti-inflammatory cytokine and at least one pro-inflammatory cytokine in the biological sample at a time following treatment with the composition;

d) determining the ratio of the at least one anti-inflammatory cytokine to the at least one pro-inflammatory cytokine in the biological sample at a time following treatment with the composition;

characterised in that a ratio as determined in step (d) from the treated biological sample greater than the same ratio determined in an untreated control biological sample tested concurrently is indicative of the composition being an inhibitor of inflammatory diseases of the bowel, and a ratio as determined in (d) is the same as or less than the untreated control biological sample ratio is indicative of the composition not being an inhibitor of inflammatory diseases of the bowel.

The methods herein are suitable for use in screening and determining clinical efficacy of treatments and compositions for the treatment of inflammatory diseases of the bowel. These diseases include inflammatory gastro-intestinal disorders, some non-limiting examples of which include irritable bowel syndrome (IBS), and IBDs such as ulcerative colitis and Crohn's disease.

Preferably, the method herein is used to determine the efficacy of treatments for irritable bowel syndrome (IBS).

The treatments herein include any treatment and/or composition for use in the treatment of inflammatory diseases of the bowel. The compositions may comprise one or more ingredients that are to have their potential efficacy in the treatment of inflammatory diseases of the bowel, preferably IBS, determined. Non-limiting examples of such compositions include anti-inflammatory drugs, probiotic compositions, new compositions and compounds not known to have efficacy in the treatment of inflammatory diseases of the bowel, compositions and compounds known to alleviate the symptoms of inflammatory diseases of the bowel including new delivery forms of known drugs useful in the treatment of inflammatory diseases of the bowel and mixtures thereof.

The *in vivo* methods of the present invention are also of use in determining the response of an individual sufferer of inflammatory diseases of the bowel to a composition useful in the treatment of inflammatory disease of the bowel. This allows the determination of the efficacy of a given treatment in an individual sufferer of inflammatory disease of the bowel, and enables physicians to monitor the progress of patients, and to determine whether to change drug type or delivery form in order to optimise the treatment of the patient. This would result in the patient receiving better treatment for their disease, and a decrease in the amount of drugs and money wasted on treatments that are ineffective on a population of sufferers of inflammatory diseases of the bowel.

Biological Sample for the In Vivo Method

The method of the present invention comprises measuring cytokine levels in a biological sample obtained from a mammalian subject both before and during or after administration of said treatment. Non-limiting examples of mammalian subjects suitable for use herein include human, simian, canine, feline, bovine, ovine, porcine, rodent subjects including murine and rat species, rabbit or equine subjects, preferably a human subject. Biological samples useful herein will be well-known to one skilled in the art. As used herein, "biological sample" includes urine, plasma, serum, saliva, tissue biopsies, cerebrospinal fluid, peripheral blood mononuclear cells with *in vitro* stimulation, peripheral blood mononuclear cells without *in vitro* stimulation, gut lymphoid tissues with *in vitro* stimulation, gut lymphoid tissues without *in vitro* stimulation, gut lavage fluids, and mixtures thereof. Preferably, the biological sample used in the *in vivo* method of the present invention comprises serum, tissue biopsies, peripheral blood mononuclear cells with *in vitro* stimulation, peripheral blood mononuclear cells without *in vitro* stimulation, and mixtures thereof, more preferably peripheral blood mononuclear cells with *in vitro* stimulation, peripheral blood mononuclear cells without *in vitro* stimulation, and mixtures thereof. Peripheral blood

mononuclear cells (PBMC) may be harvested from EDTA-treated, non-coagulated venous blood using methods known to those skilled in the art, such as Ficoll-Hypaque density centrifugation. More preferably still, the *in vivo* method of the present invention utilizes a biological sample comprising peripheral blood mononuclear cells with *in vitro* stimulation. As used herein, "peripheral blood mononuclear cells" with or without *in vitro* stimulation includes freshly harvested PBMC, whole cell homogenates of freshly harvested PBMC, extracted protein fractions of freshly harvested PBMC, mRNA transcripts from freshly harvested PBMC, tissue culture medium supernatants of freshly harvested PBMC, frozen PBMC, whole cell homogenates of frozen PBMC, extracted protein fractions of frozen PBMC, mRNA transcripts from frozen PBMC, tissue culture medium supernatants of frozen PBMC, *in vitro* cultures of harvested PBMC, whole cell homogenates of *in vitro* cultures of harvested PBMC, extracted protein fractions of *in vitro* cultures of harvested PBMC, mRNA transcripts from *in vitro* cultures of harvested PBMC, tissue culture medium supernatants of *in vitro* cultures of harvested PBMC, and mixtures thereof.

As used herein "*in vitro* stimulation" includes the stimulation of biological samples outside of the donor's body, typically in a laboratory tissue culture setting. Preferably, the stimulus comprises a mitogen, probiotic, anti-CD3 molecules known to those skilled in the art, and mixtures thereof. More preferably, the stimulus comprises a mitogen, probiotic, and mixtures thereof.

As used herein, "mitogen" includes materials that are capable of inducing cell division in a high percentage of T or B cells. Suitable of examples of mitogens useful herein include lectins, bacterial lipopolysaccharides, super-antigens and mixtures thereof. As used herein, "super-antigen" includes materials that can bind to residues in the V (variable) domain of the T-cell receptor and to residues in class II MHC molecules outside of the antigen-binding cleft, even when the T-cell receptor does not recognise the antigenic peptide bound to the class II MHC molecule. Suitable examples of super-antigens useful herein include staphylococcal enterotoxins, toxic shock syndrome toxin 1, and mixtures thereof. Preferably, the mitogen comprises lectins, bacterial lipopolysaccharides, and mixtures thereof. Suitable examples of lectins useful herein include concanavalin A (isolated from Jack beans), phytohemagglutinin (isolated from kidney beans), pokeweed mitogen (isolated from pokeweed) and mixtures thereof, preferably phytohemagglutinin (PHA). Suitable examples of bacterial lipopolysaccharides useful herein include *Escherichia coli* (*E. coli*) 0111:B4, *E. coli* 055:B5, *E. coli* K-235 (all available from Sigma (St Louis, MO)), *Salmonella Minnesota*, *Salmonella typhimurium*, *Shigella flexneri*, *Klebisella pneumonia*, *Pseudomonas aeruginosa*, and mixtures thereof.

Probiotics are micro-organisms, or processed compositions of micro-organisms which beneficially affect a host. How probiotics beneficially affect the host is unknown. For the purpose of the present invention, "probiotics" is further intended to include the metabolites generated by the micro-organism during a fermentation process, if they are not separately indicated. These metabolites may be released to the medium of fermentation, or they may be stored within the micro-organism. As used herein "probiotic" also includes bacteria, bacterial homogenates, bacterial proteins, bacterial extracts, bacterial supernatants, and mixtures thereof, that perform beneficial functions for the host when given at a therapeutic dose. Therefore, yeasts, moulds and bacteria may be included. EP 0862863 lists some examples of probiotics presently known. Suitable examples of probiotics useful herein comprise strains of *Bifidiobacterium longum infantis* (NCIMB 35624) *Lactobacillus johnsonii* (CNCM I-1225), *Bifidobacterium lactis* (DSM20215), *Lactobacillus paracasei* (CNCM I-2216), and mixtures thereof. Further non-limiting examples of probiotics useful herein are described in WO 03/010297 A1, WO 03/010298 A1, WO 03/010299 A1 (all published February 6, 2003 and assigned to Alimentary Health Ltd).

Biological Sample for the In Vitro Method

The *in vitro* method of the present invention utilises a biological sample comprising at least one gut-derived cell type. Use of the gut-derived cell type in the *in vitro* method has been found to be advantageous over prior art *in vitro* methods involving other tissues such as, for example, peripheral blood mononucleocytes. Without being bound by theory, this is though to be because the interaction between the composition for treating inflammatory diseases of the bowel to be screened and the gut is more indicative of what is occurring *in vivo*. It has been found that the *in vitro* screening method herein more readily identifies potential treatments of inflammatory diseases of the bowel, and has an improved correlation with clinical efficacy. Without being bound by theory, this is thought to be due to the fact that in inflammatory diseases of the bowel, it is the gut tissue that plays a central role in mediating the inflammatory response, and therefore those compositions that beneficially alter the cytokine ratio of gut-derived cell types *in vitro* are more likely to have an effect *in vivo*.

As used herein "gut-derived cell type" includes cell types and strains that have their origin in a mammalian gut or gut-associated tissue, said tissue including the stomach, duodenum, jejunum, ileum, lymphoid, mesentery, axillary, inguinal, popliteal, myeloid, cecum, colon, appendix and rectum. Furthermore, such cell types also include cells freshly isolated from said tissues, and cell types that have been routinely cultured *in vitro* following isolation from the above tissues, including non-immortalized cell lines, carcinogenic and otherwise immortalized cell lines. Preferably, the *in vitro* method utilises a biological sample comprising gut-associated lymphoid tissues, either freshly isolated, or *in vitro* cell cultures thereof. More preferably, the biological

sample comprises mesenteric lymph node cells, either freshly isolated, or *in vitro* cell cultures thereof.

Furthermore, the *in vitro* method of the present invention may additionally comprise the further step of stimulating the biological sample prior to step (c) above. As used herein "*in vitro* stimulation" includes the stimulation of biological samples outside of the donor's body, typically in a laboratory tissue culture setting. Preferably, the stimulus comprises a mitogen, probiotic, anti-CD3 molecules known to those skilled in the art, and mixtures thereof. More preferably, the stimulus comprises a mitogen, probiotic, and mixtures thereof.

As used herein, "mitogen" includes materials that are capable of inducing cell division in a high percentage of T or B cells. Suitable of examples of mitogens useful herein include lectins, bacterial lipopolysaccharides, super-antigens and mixtures thereof. As used herein, "super-antigen" includes materials that can bind to residues in the V (variable) domain of the T-cell receptor and to residues in class II MHC molecules outside of the antigen-binding cleft, even when the T-cell receptor does not recognise the antigenic peptide bound to the class II MHC molecule. Suitable examples of super-antigens useful herein include staphylococcal enterotoxins, toxic shock syndrome toxin 1, and mixtures thereof. Preferably, the mitogen comprises lectins, bacterial lipopolysaccharides, and mixtures thereof. Suitable examples of lectins useful herein include concanavalin A (isolated from Jack beans), phytohemagglutinin (isolated from kidney beans), pokeweed mitogen (isolated from pokeweed) and mixtures thereof, preferably phytohemagglutinin (PHA). Suitable examples of bacterial lipopolysaccharides useful herein include *Escherichia coli* (*E. coli*) 0111:B4, *E. coli* 055:B5, *E. coli* K-235 (all available from Sigma (St Louis, MO)), *Salmonella Minnesota*, *Salmonella typhimurium*, *Shigella flexneri*, *Klebisella pneumonia*, *Pseudomonas aeruginosa*, and mixtures thereof.

Probiotics are micro-organisms, or processed compositions of micro-organisms which beneficially affect a host. How probiotics beneficially affect the host is unknown. For the purpose of the present invention, "probiotics" is further intended to include the metabolites generated by the micro-organism during a fermentation process, if they are not separately indicated. These metabolites may be released to the medium of fermentation, or they may be stored within the micro-organism. As used herein "probiotic" also includes bacteria, bacterial homogenates, bacterial proteins, bacterial extracts, bacterial supernatants, and mixtures thereof, that perform beneficial functions for the host when given at a therapeutic dose. Therefore, yeasts, moulds and bacteria may be included. EP 0862863 lists some examples of probiotics presently known. Suitable examples of probiotics useful herein comprise strains of *Bifidiobacterium longum infantis* (NCIMB 35624) *Lactobacillus johnsonii* (CNCM I-1225), *Bifidobacterium lactis* (DSM20215), *Lactobacillus paracasei* (CNCM I-2216), and mixtures thereof. Further non-

limiting examples of probiotics useful herein are described in WO 03/010297 A1, WO 03/010298 A1, WO 03/010299 A1 (all published February 6, 2003 and assigned to Alimentary Health Ltd).

Cytokines

The methods of the present invention comprise the step of measuring at least one anti-inflammatory and at least one pro-inflammatory cytokine levels in a biological sample obtained from a mammalian subject. In the *in vivo* method, said samples are collected both before and after treatment with the composition of interest. It is known to those skilled in the art that cytokines are pleiotropic, and express multiple biologically overlapping activities. Accordingly, it should be understood that although the cytokines useful herein are categorized by their inflammatory action in the present system, some such ingredients can in some instances provide more than one immune response action. Therefore, the classifications herein are made for the sake of convenience.

Anti-inflammatory cytokines useful in the present invention comprise those known in the art. Non-limiting examples of anti-inflammatory cytokines useful herein include interleukin-4, interleukin-5, interleukin-13 interleukin-10, transforming growth factor-□, and mixtures thereof. Preferred anti-inflammatory cytokines for use in the present invention include interleukin-10 (IL-10: accession number: CAA55201; GI accession ID: 14625940), transforming growth factor-β isoforms 1, 2, 3 and 4, and mixtures thereof. Pro-inflammatory cytokines useful in the present invention comprise those known in the art. Non-limiting examples of pro-inflammatory cytokines useful herein include interleukin-2, heterodimeric interleukin-12, tumour necrosis factor-α, tumour necrosis factor-□, interferon-γ, and mixtures thereof. Preferred pro-inflammatory cytokines useful herein include heterodimeric interleukin-12 (IL-12: accession number: chain A; 1F45A, chain B; 1F45B; GI accession ID: chain A; 1479640; chain B; 1479641), tumour necrosis factor-α (TNF: accession number: AAC03542; GI accession ID: 2905634), interferon-γ (INF: accession number: NP_000610; GI accession ID: 10835171), and mixtures thereof.

Means for Measuring Levels

According to the method of the present invention, the levels of at least one anti-inflammatory cytokine and at least one pro-inflammatory cytokine in the biological sample are measured. Means for measuring the levels of anti-inflammatory or pro-inflammatory cytokines, or both, comprise methods known to those skilled in the art. The levels of said anti-inflammatory and said pro-inflammatory cytokines may be measured by measuring mRNA expression or protein expression, as is known to one skilled in the art. Non-limiting examples of such methods include immunosorbent assays, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), multiplexed ELISAs on microarray platforms, multiplexed ELISAs using coded microspheres coupled with a flow cytometer detection systems, bioassays, Western

blots, chromatograph-based separation systems, RT-PCR, competitive reverse transcription PCR, Northern blots, gene arrays, direct measurement of m-RNA, and mixtures thereof, preferably ELISAs, RIAs, multiplexed ELISAs using coded microspheres coupled with a flow cytometer detection systems, chromatograph-based separation systems, western blots, and mixtures thereof. More preferably the means comprises multiplexed ELISAs using coded microspheres coupled with a flow cytometer detection systems. Suitable ELISAs for use in the method of the present invention comprise those known to those skilled in the art, non-limiting examples of which include direct ELISAs, indirect ELISAs, direct sandwich ELISAs, indirect sandwich ELISAs, and mixtures thereof. A non-limiting example of commercially available multiplexed ELISAs using coded microspheres coupled with a flow cytometer detection systems suitable for use herein is the LINCOplex kit assay available from Linco Research Inc., Missouri, USA coupled with a BIOPLEX BEAD FLOW CYTOMETER™ from Bio Rad GmbH.

Ratio

The methods of the present invention comprise the steps of determining the ratio of at least one anti-inflammatory cytokine to at least one pro-inflammatory cytokine. In the *in vivo* method, these levels are determined both before administration of the treatment, and determining the same ratio either during the treatment or following completion of the treatment. In the *in vitro* method, the levels of at least one anti-inflammatory cytokine to at least one pro-inflammatory cytokine are determined in the treatment sample, and an untreated control biological sample tested concurrently. The control biological sample must be derived from the same biological sample as that used for the treatment group, and must be run as an untreated control using methods and principles known to those skilled in the art. As used herein, the ratio of anti-inflammatory cytokine to pro-inflammatory cytokine means the level of anti-inflammatory cytokine divided by the level of pro-inflammatory cytokine. Such ratio can be described by the formula:

$$\text{Ratio} = \frac{\text{Level of Anti-Inflammatory Cytokine}}{\text{Level of Pro-Inflammatory Cytokine}}$$

Without wishing to be bound by theory, it is believed that according to the present invention, if the ratio as determined herein increases after or during treatment, when compared with the ratio determined prior to the commencement of treatment or the untreated control biological sample, then the treatment is considered to be an inhibitor of inflammatory diseases of the bowel. The ratio herein can be determined using any anti-inflammatory cytokine, and any pro-inflammatory cytokine herein. Preferably, the ratios useful herein include the ratio IL-10/IL-12, the ratio IL-10/IFN-□, the ratio of □L1/TNF-□, and mixtures thereof. Without wishing to be bound by theory, it is believed that the specific ratios described herein are pivotal to the progression or remission of inflammatory diseases of the bowel, and therefore alterations in the

ratios herein are indicative of the inhibition or promotion of disease effects by treatments being investigated. It appears that individuals with inflammatory diseases of the bowel have a skewed cytokine profile that is indicative of an inflammatory condition. Similarly, the cytokines used herein that demonstrate the greatest change following treatment with inhibitors of inflammatory diseases of the bowel indicate that sufferers of these diseases have PBMC that are biased towards greater Th-1 activity, altering the normal Th-1/Th-2 cytokine balance. It has surprisingly been found that by increasing the ratios described herein, the symptoms of inflammatory diseases of the bowel can be alleviated. Without wishing to be bound by theory, it is believed that this is due to the fact that by increasing these ratios in sufferers of inflammatory diseases of the bowel, the treatment brings the ratios back up towards, or close to, those levels found in healthy subjects.

Kits

According to the present invention, kits are provided for carrying out the method of the present invention. Preferably the kits comprise a first measuring element or system for measuring at least one anti-inflammatory cytokine in a biological sample from a mammalian subject before treatment and at at least one time point after or during treatment, a second measuring element or system for measuring at least one pro-inflammatory cytokine in a biological sample from said mammalian subject before treatment, and at at least one time point after or during treatment, wherein the change in ratio of anti-inflammatory to pro-inflammatory cytokine after administration of the treatment can be determined. Such measuring elements or systems may include those known to one skilled in art, non-limiting examples of which include immunosorbent assays, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), multiplexed ELISAs on microarray platforms, multiplexed ELISAs using coded microspheres coupled with a flow cytometer detection systems, bioassays, Western blots, chromatograph-based separation systems, RT-PCR, competitive reverse transcription PCR, Northern blots, gene arrays, direct measurement of m-RNA, and mixtures thereof, preferably ELISAs, RIAs, multiplexed ELISAs using coded microspheres coupled with a flow cytometer detection systems, chromatograph-based separation systems, western blots, and mixtures thereof, more preferably multiplexed ELISAs using coded microspheres coupled with a flow cytometer detection systems. The kits of the present invention may further comprise means for obtaining the biological sample from the subject. Such means may comprise venous blood collection tubes and devices for collecting said blood, sample tubes for urine or saliva, means for separating PBMCs from venous blood, and mixtures thereof.

Furthermore, the kits of the present invention may comprise instructions for use. The instructions for use may comprise language indicating how to determine the ratio according to the present invention, and/or language indicating what the results of the performed method mean. A

non-limiting example of such instructions includes language indicating that an increase in the ratio of anti-inflammatory cytokine to pro-inflammatory cytokine is indicative of an inhibitor of inflammatory diseases of the bowel.

Examples

In Vivo Methodology

Ten healthy adults and 13 self-reported irritable bowel syndrome (IBS) patients were fed daily with probiotic preparation of 100 ml milk containing 10^8 colony forming units (CFUs)/ml of *Bifidobacterium infantis* 35624 (a potential treatment of IBS) for 3 weeks. Venous blood was drawn from each subject before and after oral feeding with the probiotic preparation. Venous blood was collected into blood collection tubes (CPT™ VACUTAINER cell separation tubes containing sodium heparin, Becton Dickinson, Franklin Lakes, NJ). PBMC were isolated from the blood using by centrifugation at 400 x *g*, and subsequently cultured *in vitro* in appropriate cell culture conditions as known to one skilled in the art. Dulbecco's Minimum Essential Media (DMEM) supplemented with 100 U/ml penicillin G/100 □g/ml streptomycin (Gibco), 2.5 □g/ml fungizone (Gibco), 292 □g/ml L-glutamine (Gibco) and 10% foetal calf serum (#1103155, Gibco) was used herein for all cell cultures except for the measurement of TGF-□wherein the foetal calf serum was left out. The isolated PBMC suspension was adjusted to a viable cell count of 1.3 x 10^6/ml and were cultured either alone in medium (no stimulation), or with 0.1 □g/ml *E.* coli 0111B4 lipopolysaccharide (Sigma, St Louis, MO), 1 □g/ml phytohemagglutinin (PHA – Sigma, St Louis, MO) or 10^7 CFU/ml whole *Bifidobacterium infantis* 35624 for 3 days. Subsequently, cell suspensions were collected into microfuge tubes and centrifuged in an MRX-152 microfuge (Tomy Tech, Palo Alto, CA) at 10 000 rpm at 4°C for 3 minutes and supernatants collected for analysis.

The quantity of cytokines in supernatants were analysed by using the commercially available LINCOplex kit assay (from Linco Research Inc., Missouri, US) in a BIOPLEX BEAD FLOW CYTOMETER ™ (Bio Rad, Hercules, CA). Twenty-five □l of culture supernatant were incubated with a panel of microsphere beads coated with antibodies specific to the cytokines interleukin-10 (IL-10), interleukin-12 (IL-12), tumour necrosis factor-α (TNF), interferon-γ (INF), and transforming growth factor-β (TGF) (LINCOplex kit assay from Linco Research Inc., Missouri, US). Bead mixtures were washed and further incubated with streptavidin-phycoerythrin at room temperature for 30 minutes. Concurrently, controls and standards, prepared in serial dilutions were also incubated with the aforementioned procedures. Bead suspensions were washed and resuspended in buffer for reading by the BIOPLEX system. The cytokine levels were quantitated in units of pg/ml. The cytokine levels in both populations and the feeding effects were analysed statistically with Student's paired t-test.

Daily oral feeding with probiotic *Bifidobacterium infantis*35624 for 3 weeks increased the ratio IL-10/IL-12 from 11.2 ± 3.9 (mean ± standard error) to 409.5 ± 95.2 in IBS patients' PBMC with *in vitro* stimulation by *Bifidobacterium infantis* 35624. Similarly, when PBMC from the IBS population were stimulated *in vitro* with other probiotics including *Lactobacillus* 299V and *Lactobacillus* GG, similar increases in the IL-10/IL-12 ratio were observed (see figure 1).

Additionally, probiotic stimulation (*Bifidobaterium*) of PBMC from IBS patients after feeding demonstrated an increase from 0.1 ± 0.0 to 2.0 ± 0.5 in the ratio IL-10/TNF-□□ similar results were also seen following stimulation with other probiotics including *Lactobacillus* 4331 and *Lactobacillus* 299V (see Figure 2). Also, stimulation with probiotic *Bifidobacterium infantis* 35624 resulted in an increase in the IL-10/IFN-□ ratio from 0.7 ± 0.1 to 14.1 ± 2.3 following treatment (see figure 3).

Furthermore, it was observed that the mean abdominal pain/discomfort decreased and the mean IL-10 to IL-12 ratio increased in those IBS patients treated with *Bifidobacterium infantis*. The negative correlation between the change in abdominal pain/discomfort and the change in IL-10 to IL-12 ratio indicatedthat the increase in IL-10 to IL-12 ratio was associated with the relief from IBS symptom of abdominal pain/discomfort (see Table 1).

In Vitro Methodology

This study was approved by the Ethics Committee of Cork University Hospital. Mesenteric lymph nodes were obtained from ten patients with active IBD (n=10: age range 19-41 (mean 32.2), 3 women and 7 men: 5 patients with CD; 5 patients with UC). All patients were prescribed and taking 5'- amino salicylic acid. All patients required surgical section because of progressive disease and failure to respond to steroids. Peripheral blood mononuclear cells (PBMCs) were obtained from patients with active inflammatory bowel disease (n=12). The age range was 20-51 (mean 28). Five women and seven men were studied, 5 patients with CD; 7 patients with UC.

Mesenteric lymph node cell (MLNC) isolation

Mesenteric lymph nodes (MLN) were selected for this study with careful regard to their anatomical location relative to areas of inflammation in the bowel. MLN selected directly drained an inflamed area of the bowel. From three patients within this study population, it was possible to obtain MLN draining segments of inflamed and non-inflamed bowel. Single cell suspensions were generated from MLN by gentle extrusion of the tissue through a 180μ mesh wire screen. Cells were washed and resuspended in DMEM (Dulbecco's modified eagle medium) containing 10% FCS (Invitrogen, Paisley, UK). Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation and resuspended at $1x10^6$ cells/ml in complete media - DMEM containing 25mM glucose, 10% FCS, 1% nonessential amino acids, 50U/ml penicillin and 50□g/ml streptomycin

(Invitrogen, Paisley, UK). These mononuclear cells are termed mesenteric lymph node cells (MLNCs).

Dendritic cell isolation

DCs from MLN and peripheral blood were isolated using identical procedures. Cells were resuspended at 5 x 10^7 cells/ ml in PBS (without Ca^{2+} or Mg^{2+}) with 4% FCS. For optimal recovery of DCs, 1mM EDTA was added to all media and cell suspensions were blocked with anti-CD32 antibodies (StemCell, Meylan, France). DCs were isolated from this cell suspension, according to the manufacturer's protocol, using a DC negative isolation kit (StemSep™ depletion cocktail, StemCell, Meylan, France). DCs were resuspended in Stemspan serum-free media (StemCell, Meylan, France) at 1x10^6 cells/ ml. Viability, determined by trypan blue exclusion, was consistently ≥ 98%. Purity of DC preparations were assessed using flow cytometry. Cells which were HLA-DR positive and CD3/CD14/CD16/CD19/CD20/CD56 negative were termed DCs. All antibodies were obtained from BD Biosciences, (Oxford, UK).

Bacterial strains

We have previously reported the selection criteria for isolation of *Lactobacillus salivarius* UCC118 (*L. salivarius*) and *Bifidobacterium infantis* 35624 (*B. infantis*). *L. salivarius* and *B. infantis* were routinely cultured anaerobically for 24-48 hours in deMann, Rogosa and Sharpe medium, MRS, (Oxoid, Basingstoke, UK) and MRS supplemented with 0.05% cysteine (Sigma, Dublin, Ireland), respectively. *Salmonella typhimurium* UK1 (*S. typhimurium*) was cultured aerobically for 18-24 hours in tryptic soya broth (Oxoid, Basingstoke, UK). Bacterial cultures were harvested by centrifugation (3,000g x 15 mins), washed with PBS and subsequently diluted to form compositions comprising final cell densities of 1x10^7, 1x10^5 and 1x10^3 colony forming units (cfu)/ ml in DMEM.

In vitro Treatment of Biological Sample

All cells were seeded in 24 well tissue culture plates (Costar, Schiphol-Rijk, Netherlands) at 1x10^6 cells/ml. MLNCs were stimulated for 72 hours with compositions comprising *L. salivarius, B. infantis* or *S. typhimurium* at 3 different bacterial concentrations, 1x10^7 CFU, 1x10^5 CFU and 1x10^3 CFU. As treatment with 1x10^7 bacteria resulted in significant stimulation of cytokine production, this bacterial concentration was used in subsequent experiments. MLNCs isolated from non-inflamed bowel were stimulated for 72 hours with these bacteria at 1x10^7 cfu. MLN-derived DCs were stimulated with *L. salivarius, B. infantis* or *S. typhimurium* for 24 hours. Untreated control biological samples were present to assess spontaneous levels of cytokine secretion. Plates were incubated in a 5% CO_2 and 37°C humidified atmosphere after which supernatants were harvested for cytokine analysis. Cytokine production was measured, according

to the manufacturer's instructions, using commercially available ELISA kits (R&D Systems, Abington, UK). Cytokines measured included TNF-α, IL-12 p40, IL-10 and TGF-β.

Statistical Analysis

Results were analysed using ANOVA analysis. Values are illustrated as the mean ± standard error mean. Statistically significant differences in cytokine production between non-stimulated cells (control) and cells stimulated with bacteria were accepted at $p < 0.05$.

Results

As can be seen from figures 4, 5 and 6, the ratio of anti-inflammatory cytokines to pro-inflammatory cytokines produced by gut-derived cell types *in vitro* following stimulation with probiotic bacteria (Bif. 35624 and Lb. 118) is greater than the same ratio produced by non-stimulated control samples. This trend, whilst not always reaching statistical significance, is indicative of the different interaction probiotic compositions, and compositions in general that may be beneficial in the treatment of inflammatory diseases of the bowel, have on the gut tissues *in vivo* compared with that of pathogenic bacteria. This is believed to be indicative of the composition being an inhibitor of inflammatory diseases of the bowel. As can be seen from figures 4, 5 and 6, stimulation with pathogenic bacteria (Salmonella) either results in no difference in the ratio of anti-inflammatory cytokines to pro-inflammatory cytokines when compared with the control, non-stimulated sample, or more typically, a reduction in said ratio. Again, whilst this difference does not always reach statistical significance, it is indicative of a composition that is not an inhibitor of inflammatory diseases of the bowel.